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Aspects of Gametophyte Development of *Dicksonia sellowiana* Hook (Dicksoniaceae): an Endangered Tree Fern Indigenous to South and Central America

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Abstract.—With the purpose of providing a basis for programs of sustainable management in the conservation of this endangered species, this paper presents morphological aspects on the gametophyte development of *Dicksonia sellowiana* (Dicksoniaceae) by light microscopy and scanning electron microscopy. *Dicksonia sellowiana* spores were germinated in Morh's nutrient solution modified by Dyer (1979) under a 16-hour photoperiod at 23 ± 2°C. To determine the best substrate for gametophyte and sporophyte development, 30 days after spore sowing filamentous gametophytes were transferred to different substrates: soil rich in organic matter; coxim (coconut fiber); sterilized typic hapludult soil (distroferric red nitosoil); and sterilized typic hapludult soil (distroferric red nitosoil) with the addition of organic compost. The best system for *D. sellowiana* growth was the red soil with the addition of compost. Fifteen days after spore sowing in mineral solution, gametophytes were filamentous. Some had attained laminar morphology and had established an oblique cell division, giving rise to the obconic cell. Laminar gametophytes were observed 30 days after spore sowing and cordate gametophytes were observed after 45 days. Mature cordate gametophytes were observed after 80–90 days. After 245 days 84.67% of gametophytes had produced sporophytes in sterilized red soil with the addition of organic compost. In typic hapludult soil, without the additional termophilic compost, sporophyte formation was delayed (development after 180 days). When gametophytes were transplanted to soil rich in organic matter they did not develop and in the "coxim" substrate, which is a substitute for the "xaxim" substrate, only filamentous gametophytes were observed at the end of the study.

Key Words.—*Dicksonia sellowiana*, gametophyte development, substrate comparison

The Atlantic Forest biome entirely occupies three Brazilian states: Espírito Santo, Rio de Janeiro and Santa Catarina, 98% of Paraná and some areas of 11 other states (IBGE, 2004; Fundação Biodiversitas, 2006). Ferns are an important plant group of the Brazilian flora. According to Tryon (1970, 1972) and Tryon and Tryon (1982), southeastern Brazil (from Minas Gerais to Rio Grande do Sul) contains about 600 fern species. Some of the Brazilian ferns are used as ornamental plants and members of the tree fern families Cyatheaceae, Dicksoniaceae and Cibotiaceae have been indiscriminately exploited through the commercialization of pots and substrate used in the production of ornamental plants (Windisch, 2002). For that reason, under-

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standing aspects of fern biology is necessary for the development of methods that may assist in their conservation and management.

In Brazil, *Dicksonia sellowiana* Hook. (Dicksoniaceae) is considered an endangered species of the Atlantic Forest biome (IBAMA, 1997). It occurs preferentially in high humidity environments and on river banks, independent of soil conditions (Fundação Biodiversitas, 2006). The stem is usually massive, ranging from 12–20 cm in diameter, arborescent, 10 m tall, basally decumbent, bearing long, dense trichomes and many fibrous roots, which may occur from the base almost to the apex. It occurs at ca. 1500–2500 m, sometimes up to 3500 m, or in Brazil at lower elevations. It occurs throughout Central America and in South America from Venezuela to Colombia, south of Bolivia, Paraguay, Uruguay and southeastern Brazil (Sehnem, 1978; Tryon 1970, 1972; Tryon and Tryon, 1982). In Brazil, it is known as 'xaxim' or 'xaxim bugio' and the trunks have been indiscriminately exploited through the commercialization of vases and substrate (Sehnem, 1978).

The understanding of fern germination and establishment is required for their "ex situ" conservation. The germination of a great number of fern spores is promoted by light (Millër, 1968) and nutrients, water and mild temperatures are implicated in the growth and development of the prothallus and in sporophyte formation (Fernández *et al.*, 1996, 1999). Several aspects of the germination of *D. sellowiana* have been studied. Fillipini *et al.* (1999) sterilized spores in a 5% solution of commercial bleach for 10 min and reported that the spores of this species reached around 88% germination at 23 ± 2°C under continuous white light, seven days after sowing in liquid mineral medium. The same authors reported that the spores stored under refrigeration remained viable for more than two years and reached 81.75% germination 10 days after spore sowing, which did not differ from the germination of recently collected spores (Fillipini *et al.*, 1999). Under 50% and 36% irradiance, the germination of *D. sellowiana* spores was delayed after 14 and 21 days, respectively, of culture compared to 20% and 5% irradiance. Higher percentages of germination (around 90%) and lower mean germination time (34 days) were observed for spores of *D. sellowiana* sterilized in a 35% solution of commercial bleach for one hour, which germinated at 20% and 5% sunlight; no statistically significant differences were observed between the two light treatments (Fillipini *et al.*, 1999; Renner and Randi, 2004). To study the possibility of long-term spore storage of *Dicksonia sellowiana* for the establishment of a germplasm bank, Rogge *et al.* (2000) stored spores in liquid nitrogen and reported that spores remained viable after being immersed in liquid nitrogen for three months. Concerning patterns of gametophyte development, Nayar and Kaur (1969) described seven different types of prothallial development in the homosporous ferns. In previous works it was reported that germination in *D. sellowiana* is of *Vittaria* type and prothallic development is of *Adiantum* type (Pérez-Garcia and Fraille, 1986).

To obtain sporophytes of *Dicksonia sellowiana* cultivated from germinated spores, Borelli *et al.* (1990) cultivated *D. sellowiana* in the soil of "xaxim" (*D. sellowiana*) trunks and observed sporophytes after six months of spore sowing.

They commented that fungal contamination was very high in all the treatments. Suzuki *et al.* (2005) cultivated *D. sellowiana* from spores and observed sporophytes that had been emerged in sterilized typic hapludult soil (red soil) with the addition of termophilic organic compost; the first sporophyte frond was observed 84 days after transplantation.

The aim of the present study was to observe gametophytes of *Dicksonia sellowiana* grown in different substrates in the laboratory to examine morphological aspects of gametophyte development using light microscopy and scanning electron microscopy in order to determine suitable conditions for their growth and development.

Material and Methods

Sporophylls of *Dicksonia sellowiana* were collected from living plants in August 1999 in Urupema, a fragment of the Atlantic Forest biome, situated between 27°57′25″S and 49°53′33″W, Santa Catarina state, Brazil. Sporophylls were air-dried in an oven at 30°C for three days on filter paper in order to induce dehiscence. The spores were removed and separated from debris by filtering through lens paper, and then were stored in glass jars under refrigeration at 7 ± 1°C.

Spores were surface-sterilized using a 20% (v/v) solution of commercial bleach (2% active chlorine), which corresponds to 0.1% of active chlorine, for a period of 30 min before filtering through sterile filter paper and washing several times with sterile distilled water. About 20 mg of sterilized spores were sown in each of 32 conical flasks containing 20 ml of Mohr's nutrient solution as presented by Dyer (1979) with the addition of 25 mg L^{-1} Benomyl to avoid fungal contamination. The flasks were plugged with two layers of autoclaved transparent commercial polypropylene film (7 × 7 cm) fixed with rubber bands. All the procedures were carried out in a laminar hood. The spores were incubated in a 16-hour photoperiod (30 μmoles quanta · m^2 · s^1) at 23 ± 2°C in January 2002.

In February 2002, the young gametophytes cultivated in liquid medium were transplanted to trays containing four types of substrates: commercial substrate rich in organic matter; commercial "coxim": substrate produced from the coconut fiber used as substitute for the soil made from the "xaxim" (*D. sellowiana* trunks); sterilized typic hapludult soil (distroferric red nitosoil) and sterilized typic hapludult soil (3 parts) with addition of termophilic organic compost (1 part) as described in Suzuki *et al.* (2005).

The soil analysis was carried out in Soil Laboratory of CIDASC (Company for Agricultural Development of Santa Catarina) (Table 1). The trays were covered with transparent film to avoid excessive water evaporation and plant dehydration. Substrate sterilization was carried out in a high power microwave oven for 20 minutes. The organic compost was produced from vegetable and fruit wastes at the University of Santa Catarina. Sporophyte emergence was scored once a week. The mean and standard deviation for each day of evaluation was calculated by Excel for Windows (Microsoft).

TABLE 1. Analysis of substratum mineral composition (CIDASC-analysis number 07462/2003).

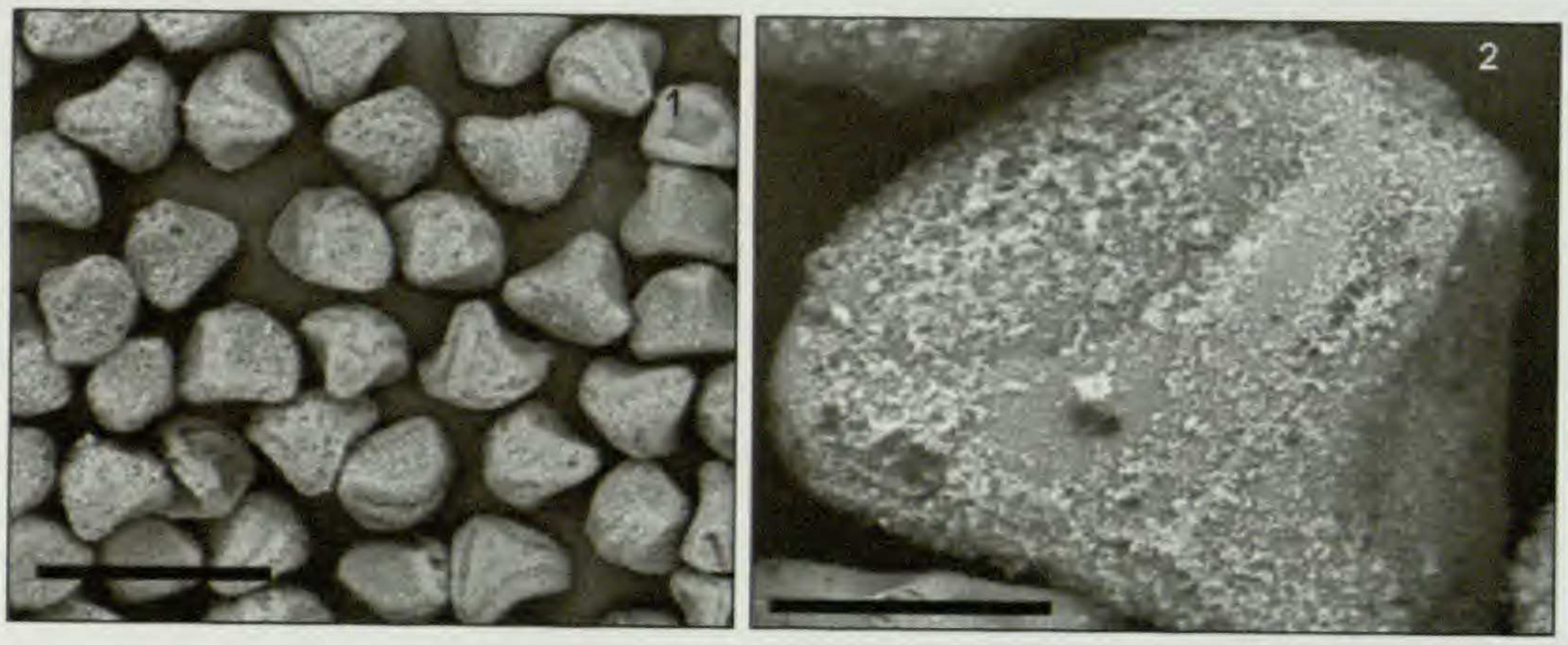
	Soil rich in organic matter	Coxim	Typic hapludult soil	Typic hapludult soil plus termophilic compost
pH	6.60	5.20	4.40	5.20
P (ppm)	> 50.00	38.30	2.60	> 50.00
K (ppm)	340.00	1204.00	95.00	450.00
% organic matter	4.30	> 10.00	0.80	0.90
% total N	0.12	0.35	0.03	0.26
Al (cmolc/l)	traces	0.3	2.2	traces
Ca (cmolc/l)	5.4	1.7	1.7	4.8
H^+, Al (cmolc/l)	2.48	1.89	8.79	3.90
CEC (cmolc/l)	13.92	9.37	11.32	12.88

Specimens were collected every 15 days from the mineral solution and from the trays containing sterilized typic hapludult soil and organic compost. For light microscopy (LM) and scanning electron microscopy (SEM), gametophytes were fixed in 2.5% glutaraldeyde in 0.1 M sodium phosphate buffer 7.2 pH. All samples were fixed in Eppendorf tubes for 3-hr, then were centrifuged for 3 min, dehydrated with an ethanol series and stored in ethanol. The samples were photographed with a Leika-MPS 30 light microscope. For LM, the samples were mounted on glass slides with ethanol. For SEM, the cordate gametophytes were dehydrated with graded ethanol (80%, 90%, 96% and 3 times in 100%). Subsequently, they were transferred to HMDS (hexamethyldesilasane) to substitute the CO_2 critical point, avoiding cell collapse (Bozzola and Russel, 1991). Dry samples were transferred to stubs and then were gold-coated with 20 nm of gold in a Baltec-CED 030. Examination was performed with a Philips-XL 30 scanning electron microscope.

RESULTS

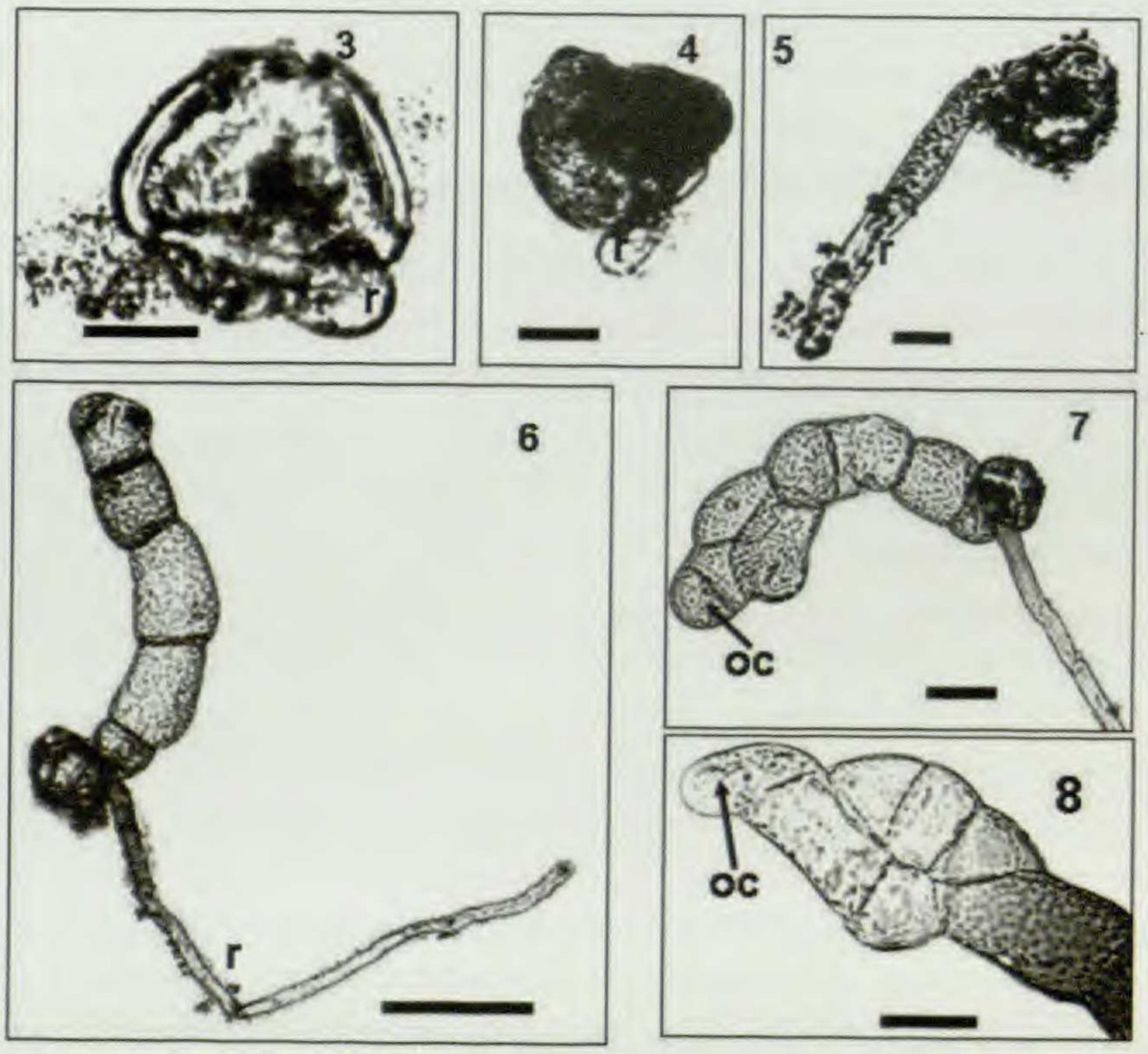
The spores of *Dicksonia sellowiana* are tetrahedral, globose and trilete; the surface is densely granulated and measure 44–68μm (Figs. 1 and 2). *Dicksonia sellowiana* germination is of the *Vittaria* type (Pérez-Garcia and Fraille, 1986). During germination, the spores become swollen and the spore coat opens. The first division was parallel to the equatorial axis of the spores; small hemispheric cells were produced and gave rise to a hyaline rhizoid that does not contain plastids; subsequently a spherical prothallial cell which is rich in chloroplasts appeared (Figs. 3–5).

Gametophytes of *D. sellowiana* were not able to develop in the soil rich in organic matter. In the coxim, only filamentous gametophytes were observed after 8 months of cultivation. In sterilized typic hapludult soil, the first sporophytes were only observed after 6 months of cultivation, but in sterilized typic hapludult soil with addition of termophilic organic compost, sporophytes were observed after less than 3 months of cultivation.

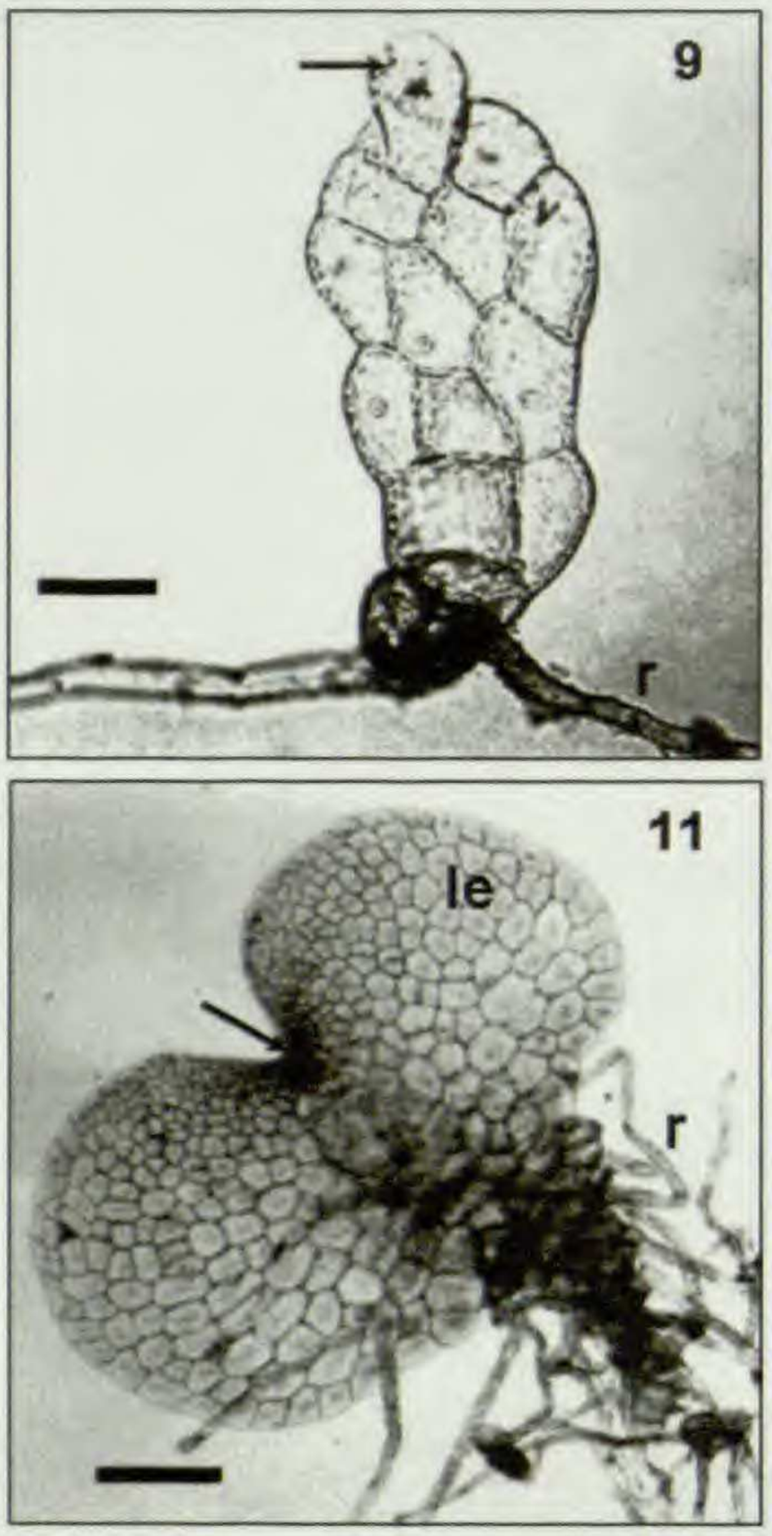


FIGS. 1–2. 1. Scanning electron micrographs of *Dicksonia sellowiana* spores. 2. Tetrahedral spores. Bar = 100 μm. 3. Detail of the spore with densely granulated surface. Bar = 20 μm.

Fifteen days after sowing, a uniseriate filament was apparent, consisting of 3–7 cells as a result of parallel divisions of the original prothallial cell. The filament cells showed abundant chloroplasts and the spore coat was still attached to the basal cell. There was only one rhizoid present (Fig. 6). A wall parallel to the axis of the filament divided the terminal cell; further division followed an oblique direction, giving rise to an obconic or meristematic cell (Figs. 7 and 8). The laminar phase of *D. sellowiana* gametophytes was observed after 30 days of spore sowing. The beginning of the cordate or heart phase with



FIGS. 3–8. Initial phase of spore germination. Bars = 20 μm. 6. Germinated spore with rhizoid. Bar = 20 μm. 7. Filamentous gametophyte (15 days). Bar = 100 μm. 8–9. Laminar gametophyte with the initial lateral divisions (15 days). Bars = 50 μm. Legend: oc – obconic cell, r - rhizoid.

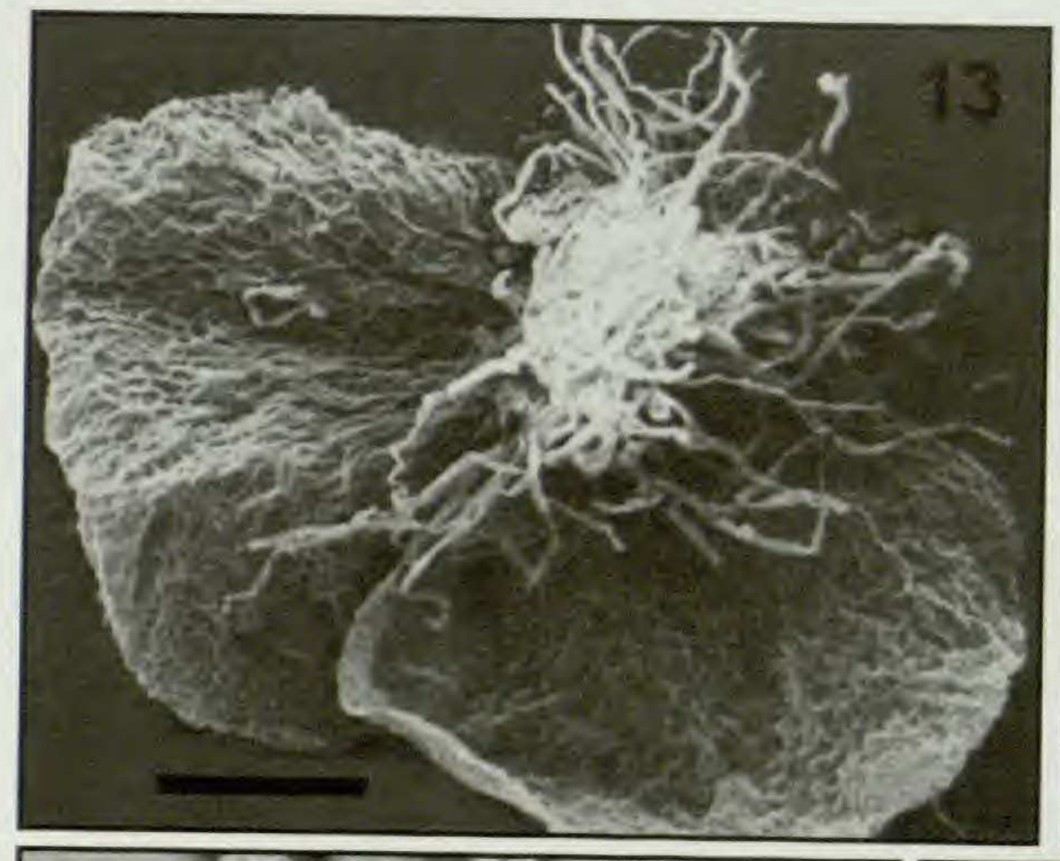


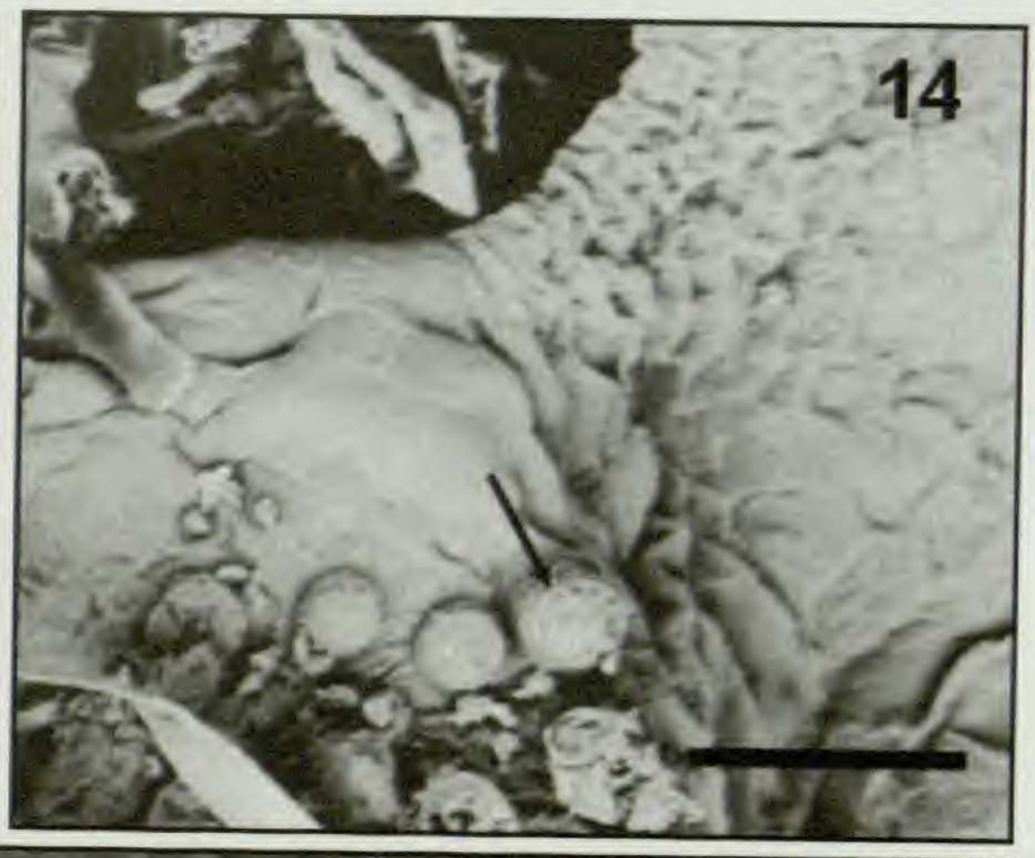
FIGS. 9–12. 9. Laminar phase (30 days) Bar = 50 µm. 10. Gametophyte spatulated (45 days). Bar = 100 µm. 11. Cordate gametophyte (75 days). Bar = 200 µm. 12. Meristematic region with archegonia (90 days). Bar = 100 µm. ac- obconic cell , amr - apical meristematic region,– ar- archegonium, le - lateral expansion, r – rhizoid.

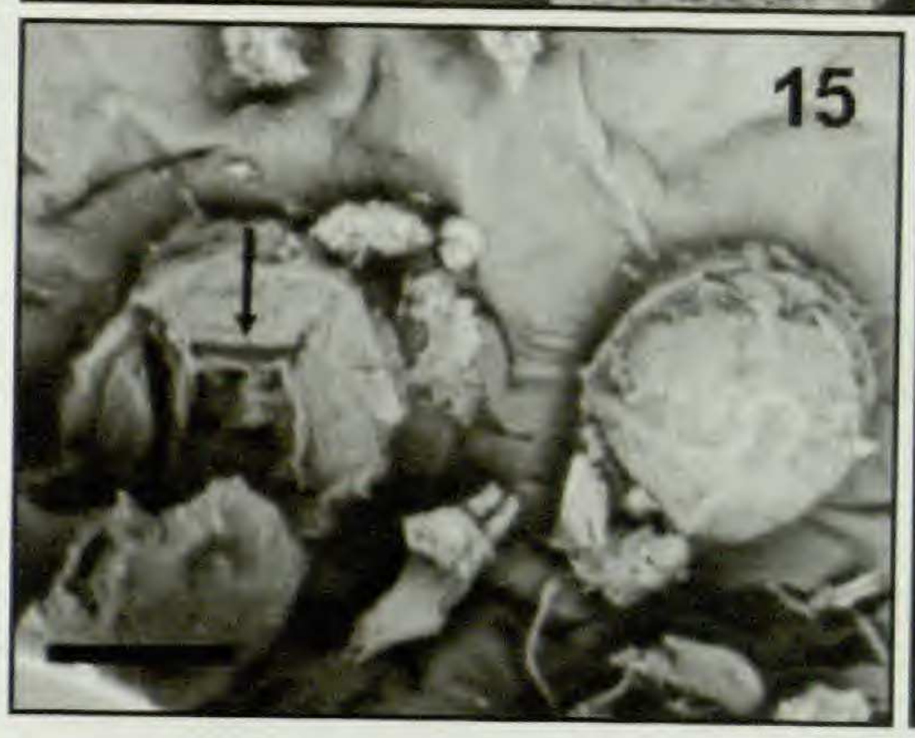
the development of the wings was observed 45 days after spore sowing and 15 days after gametophyte transplantation to the sterilized typic hapludult soil with addition of termophilic organic compost. After 75 days of spore sowing, gametophytes presented the heart shape (Figs. 9–11) and 90 days after spore sowing archegonia were present (Figs. 12 and 13). The archegonia grew from surface cells of the apical meristem, were bottle-shaped, and presented four rows of 4–5 exposed neck cells and the neck canal inside (Figs. 13–16). Antheridia were not observed in this SEM prepared material. The gameto- phytes did not bear trichomes.

DISCUSSION

The surface *of Dicksonia sellowiana* spores are granulated or reticulate with strands of fused spheres surrounding somewhat depressed areoles as observed by Tryon and Tryon (1982). The pattern of spore germination found for *Dicksonia sellowiana* was the *Vittaria* type (Nayar and Kaur 1971), which was described by Pérez-Garcia and Fraille (1986). The prothallial development of *Dicksonia sellowiana* is of the *Adiantum* type, described by Nayar and Kaur







FIGS. 13–16. Scanning electron micrographs of *Dicksonia sellowiana* gametophytes. 13. General gametophyte view. Bar = 500 μm. 14. Aspects of inferior face bearing archegonia. Bar = 100 μm. 15. Detail of an archegonia. Bar = 20 μm.

(1969) and Pérez-Garcia and Fraille (1986). In this type of gametophyte development, spore germination results in a uniseriate, slender germ filament, which is generally 3–7 cells long. The terminal cell and one or two cells behind it divide longitudinally to form a prothallial plate. The division of the terminal cell is often by the formation of a wall oblique to the long axis of the filamentous gametophyte, and soon a second oblique wall delimits a central obconical meristematic cell. By the activity of the obconical cell, a spatulated prothallial plate, without trichomes, is formed in which the apex gradually becomes notched. Examples of species from the Dicksoniaceae that show the *Adiantum* type protallial development are *Lophosoria quadripinnata* (J. F. Gmel) C. Chr. (Pérez-García *et al.*, 1995) and *Lophosoria quadripinnata* var.*contracta* (Mendoza *et al.*, 1997).

The laminar phase was observed after 30 days of cultivation for *Dicksonia sellowiana* and the heart shaped gametophytes presented archegonia 90 days after spore sowing, but they did not present antheridia. Conversely, Pérez-Garcia and Fraille (1986) observed gametophytes of *D. sellowiana* bearing antheridia only after 170 days cultivation, but they did not observe gametophytes bearing archegonia. The same authors analyzed gametophyte development only under light microscopy and did not analyze time to

sporophyte formation and percentage of sporophyte formation in different soils. They found filamentous amorphous gametophytes that were not observed in the present work. The gametophyte of *Lophosoria quadripinnata* was spatulate after 156 days of culture; antheridia were observed after 72 days, but archegonia were seen only after 270–285 days (Pérez-García *et al.*, 1995). Additional analyses are necessary to show how sexual expression of *D. sellowiana* is affected by spore density in laboratory conditions.

In Santa Catarina State (Brazil) there is a predominance of clay soils, cambisoils, laterites and nitosoils (IBGE, 2005). In the experimental conditions carried out in this study, the typic hapludult soil (distroferric red nitosoil) with the addition of termophilic organic compost was the most suitable for *Dicksonia sellowiana* development, and the first sporophytes in this substrate were observed 84 days after spore sowing. Atlantic Forest soils are poor in nutrients and the accumulation of chemical elements in cells is one of the ways tropical species tolerate low-nutrient soil (IBGE 2004; Fundação Biodiversitas, 2006). Litterfall is a fundamental component of nutrient cycling, and it is the main means of transferring organic matter and mineral elements back to the soil surface (De França *et al.*, 2007; Moraes *et al.*, 1999). The termophilic organic compost was added in order to simulate the litter in this substrate. This substrate has a low pH and high levels of N, P, K and Ca. In contrast, when Borelli *et al.* (1990) cultivated gametophytes of *Dicksonia sellowiana* in the soil made of "xaxim" trunks, the first sporophytes were observed only after six months of cultivation and the authors observed 100% contamination in their cultures and around 75% of gametophytes produced sporophytes. Therefore, the time to sporophyte formation, the percentage of sporophyte formation, and the prevention of contamination were improved in the present work. Edaphic parameters were analyzed for several fern species to elucidate their habitats including nutritional requirements and the majority of them prefer acidic soils, as does *D. sellowiana* (Carlson, 1979; Graves and Monk, 1982; Whitter and Moyroud, 1993; Ranal, 1995). The time to sporophyte emergence is quite variable among fern species; *Lophosoria quadripinnata* formed sporophytes after 36 months cultivation (Pérez-García *et al.*, 1995).

The typic hapludult soil with added termophilic organic compost employed in this work was also useful for sporophyte emergence, which was observed less than three months after cultivation as observed in previous work (Suzuki *et al.*, 2005).

Information provided in this paper certainly will be useful for *D. sellowiana* management in green houses as part of conservation strategies. Plantlets of *Dicksonia sellowiana* can be easily obtained following the protocol presented here. This methodology can be used for the establishment of germplasm banks with the purpose of preserving the species in botanical gardens and to maintain its genetic variability.

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